Repair of 254 nm Ultraviolet-Induced (6-4) Photoproducts: Monoclonal Antibody Recognition and Differential Defects in Xeroderma Pigmentosum Complementation Groups A, D, and Variant

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Repair kinetics of ultraviolet (UV) light-induced (6-4) photoproducts in xeroderma pigmentosum complementation group A, D, and variant cells were studied by the enzyme-linked immunosorbent assay (ELISA) using a specific monoclonal antibody raised against (6-4) photoproducts, together with unscheduled DNA synthesis (UDS) and loss of T4 endonuclease V-susceptible sites (ESS). Group A XP35KO cells completely failed to repair both ESS (cyclobutane pyrimidine dimers) and antibody-recognizing (6-4) photoproducts until tested 24 h after irradiation, and had 2% early-time UDS. Group D XP43KO cells showed about 10% removal of both (6-4) photoproducts and ESS in 24 h, despite showing a residually higher level of 40% early-time and cumulative UDS. Thus, the results substantiated the extreme UV hypersensitivity of XP group A and D cells. However, XP52KO variant cells exhibited the normal level of UDS and ESS loss, but a slightly reduced repair of antibody-recognizing (6-4) photoproducts at 6 and 12 h after irradiation, which may account for a small UV hypersensitivity of the XP variant cells. *J Invest Dermatol 93:703–706, 1989*

Xeroderma pigmentosum (XP) is a rare, autosomal recessive disease characterized by higher photosensitivity of the skin and a high incidence of skin malignancies in the sunlight-exposed areas [1–3]. XP cells are defective in the repair of UV damage and chemically induced DNA damage [4–5]. At present, XP comprises nine complementation groups (A through I) defective in the excision of UV damage and a variant group proficient in the cyclobutane dimer excision [6–8]. In growing cells, there is a good correlation between the extent of DNA repair defect and UV hypersensitivity in terms of post-UV, colony-forming ability [9].

254 nm UV induces a variety of DNA lesions, of which cyclobutane pyrimidine dimers are the major photoproducts [10]. The (6-4) photoproducts may account for as much as 30% of the total products and are highly mutagenic [11]. Recently, Cleaver et al [12] have indicated the significant differential contribution of cyclobutane and (6-4) dimers, such that a particular group A XP12RO revertant with normal UV sensitivity and chromosomal DNA mutagenesis can repair (6-4) photoproducts normally but not cyclobutane dimers. This further implies that (6-4) photoproducts may play a major role in the lethal and mutagenic effects of UV. Using a polyclonal antibody specific to (6-4) photoproducts, Mitchell et al [13] have shown that in addition to defective repair of cyclobutane dimers, XP-A, C, and D cells have negligible repair of (6-4) photoproducts, although normal cells remove 75% within 4 h at a rate faster than that for cyclobutane pyrimidine dimers. Francis and Regan [14] have suggested that defective repair of 313 nm sensitive non-cyclobutane-dimer lesions may be responsible for an enhanced UV sensitivity of XP variant cells. On the other hand, Mitchell et al [15] have reported that XP variant cells are proficient in the repair of (6-4) photoproducts by use of a specific polyclonal antibody. Mori et al [16] have recently established a monoclonal antibody 64M-1 that specifically recognizes the (6-4) photoproducts in the T-T and T-C sequences but not cyclobutane dimers. Further, this antibody is cross-reactive to both T(6-4)T and T(6-4)C products with a more preferential recognition of the former and has affinity to (6-4) dinucleotides [16].

In the present study we have looked at the repair of 254-nm UV-induced (6-4) photoproducts from the DNA of representative normal human, XP-A, D, and variant fibroblasts by applying ELISA (enzyme-linked immunosorbent assay) using the specific monoclonal 64M-1 antibody raised against (6-4) photoproducts.

MATERIALS AND METHODS

Cell Strains and Culture The diploid human skin fibroblast strains used were normal, XP35KO (XP-A: newly assigned by complementation test), XP43KO (XP-D) [17], and XP52KO (XP

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Abbreviations:
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ESS: T4 endonuclease V-susceptible sites
PBS: phosphate buffered saline
(6-4) photoproduct: 6-4′-(pyrimidine-2′-one)-pyrimidine class of ultraviolet induced photoproducts
SDS: sodium dodecyl sulfate
Tris-HCl: tris (hydroxymethyl) aminomethane hydrochloride
UDS: unscheduled DNA synthesis
XP: xeroderma pigmentosum

variant: an elder brother of the previous XP3KO variant [18]). Fibroblasts between passages 5 and 15 were used for experiments. Cells were cultured in Eagle's minimum essential medium (MEM, Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. All strains were mycoplasma-free.

Time Kinetics of Unscheduled DNA Synthesis (UDS) After UV Irradiation Cells were plated on two 11 × 32 mm coverslips in 6-cm dishes (Falcon 3002) and cultured for 2 d. Cells were washed twice with phosphate buffered saline (PBS), irradiated with 10 J/m² of 254 nm UV light, and continuously incubated in 370 kBq (10 μCi)/ml of [*Me*-³H]thymidine [specific activity; 1702 GBq (46 Ci)mmol, Amersham] for various lengths of time (3 to 24 h) in the absence of hydroxyurea, as described previously [19]. The cells were fixed in acetic acid-methanol (1:3). The coverslips mounted on slides were processed for autoradiography by applying a Sakura NR-M2 liquid emulsion (Konishiroku Photo Ind., Tokyo) and exposed in the dark at 4°C for 5 d. Following development and staining with Giemsa, the average number of grains per cell as a measure for cumulative UDS was calculated from grain counts over 100 lightly labeled nuclei. Early-time UDS (Table I) was from the [³H]thymidine labeling for the initial 3 h.

UV Irradiation and DNA Isolation Cells were seeded in five 10-cm dishes (Falcon 3003) at a density of 1 × 10⁶ per dish and cultured for 2 d. Cells were washed twice with PBS and irradiated with 254-nm UV light. For the UV dose-(6-4) photoproducts relation, we applied a dose range of 0 to 40 J/m². For the time-repair relation, the zero time dose was fixed to 40 J/m². Immediately after irradiation or after further incubation for desired lengths of time up to 24 h, cells were lysed in 1 ml of 1% SDS-STE (100 mM NaCl, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) per dish, and collected. The lysates were digested with 200 μg/ml of Proteinase K (Merck) for 16 h at 37°C. DNA was extracted by the phenol/*iso*amylalcohol-chroloform procedure. The ethanol precipitates were dried in vacuo, dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5), and treated with 50 μg/ml of pancreatic RNase at 37°C for 1 h. After deproteinization, purified cellular DNA (sample) was precipitated and dissolved at a concentration of 100 μg/ml in 10 mM PBS (pH 7.4). DNA isolated from non-irradiated cells was served as the control sample.

The 64M-1 Monoclonal Antibody for (6-4) Photoproducts and ELISA Mori et al [16] have recently established the 64M-1 monoclonal antibody, which specifically recognizes UV-induced (6-4) photoproducts. ELISA was employed for the detection of production and repair of (6-4) photoproducts, as described below. Briefly, wells of 96-well polyvinyl chloride flat-bottom microtiter plates were coated with 1% protamine sulfate for 2 h at 37°C and tightly bound with the standard antigen of 50 ng/50 μl of 2 kJ/m² 254 nm UV-irradiated double-stranded calf thymus DNA by incubating wells with the antigen in PBS (pH 7.4) for 20 h at 37°C. Non-specific binding sites were blocked with 1% newborn calf serum in PBS. For competition with sample DNA, wells were incubated with a mixture of 0.1 ml of the 64M-1 monoclonal antibody and 2 μg excess of single-stranded DNA (ssDNA) obtained from unirradiated and UV-irradiated normal, XP-A, XP-D, and XP variant cells. After repeated washes with PBS, 0.1 ml of the affinity purified gout anti-mouse IgG/peroxidase conjugate was reacted for 90 min at 37°C, followed by a reaction with 0.1 ml of 0.04% *o*-phenylene diamine-0.02% H_2O_2 in 20 mM citrate buffer (pH 5.0) for 30 min at 37°C. A_{490} (absorbance at 490 nm) was measured spectrophotometrically. Percent inhibition of the 64M-1 binding to the standard antigen (see above) by the competitive inhibitor of sample DNA was calculated as

$$\text{Percent inhibition} = \left(1 - \frac{A_{490} \text{ with inhibitor DNA}}{A_{490} \text{ without inhibitor DNA}}\right) \times 100$$

Other Methods The procedures for clonogenic UV survival and T4 endonuclease V-susceptible sites (ESS) are described in ref 20.

RESULTS AND DISCUSSION

XP35KO (A) and XP43KO (D) cells showing mean lethal doses (D_0) of 0.35 and 0.7 J/m², respectively, in the clonogenic survival curve were 15 and 7 times as sensitive to the lethal effect of 254 nm UV as normal cells (D_0 = 5.0 J/m²) (Table I). Such different UV hypersensitivities of XP-A and XP-D cells were correlated with 0% and 10% relative losses of T4 endonuclease V-susceptible sites (ESS) or cyclobutane dimers, respectively, in 24 h following 10 J/m² 254 nm UV (Table I), as described previously [2,20–21]. The majority of XP-D strains show 20%-50% early-time UDS of normal cells [1,2,21,23,24]. The present XP43KO (D) cells had the typical UDS value of 40% for the initial 3 h [17], which was much higher than 10% of the 24-h ESS loss (Table I). Cumulative UDS obtained by continuous [³H]thymidine labeling in 10 J/m²-irradiated XP43KO (D) cells also followed a proportionately lower increase at a 40% level of normal cells during the entire post-UV period of 24 h (Fig 1), although normal [19] and XP52KO variant cells (Fig 1) had similar early rapid and later slow UDS responses. The origin of a curious high-UDS behavior of XP-D cells despite a severe excision defect is as yet unknown, although there remains a possibility of either nick translation at damage sites without their loss or proficient repair of non-dimer lesions.

Cleaver et al [12] have recently shown from a study of an XP-A revertant that 254 nm UV-induced (6-4) photoproducts are highly lethal and chromosomally mutagenic, compared with cyclobutane dimers. A different role of cyclobutane and (6-4) dimers in UV mutagenesis in pZ189 plasmid was also distinguished using *Escherichia coli* photolyase. A 90% removal of cyclobutane dimers by photolyase reduced the plasmid killing by 75% and UV mutagenesis by 80% [11]. The above two results suggest a differential contribution of chromosomal and plasmid DNA damage of cyclobutane and (6-4) dimers to biologic end points. In this connection, we undertook to estimate the production and repair of (6-4) photoproducts in human cells.

Table I. UV Sensitivity and Repair Characteristics

	Early-time UDS[a]	UV-survival parameters[b] n	D_0 (J/m²)	%ESS Loss[c]		% Loss of 64M-1 binding sites[d] at 6 h	24 h
Normal	100	1.5	5.00	100		80	>95
XP35KO (A)	2	1.0	0.35	0		0	0
XP43KO (D)	40	1.0	0.70	10		5	10
XP52KO (Variant)	100	1.0	4.40	100	50	60	>95

[a] Autoradiographic UDS after a 3-h [³H]thymidine labeling following 10 J/m², relative to control.
[b] See Ref 17.
[c] Disappearance of T4 endonuclease V-susceptible sites 24 h after 10 J/m², relative to control.
[d] From Fig 3.

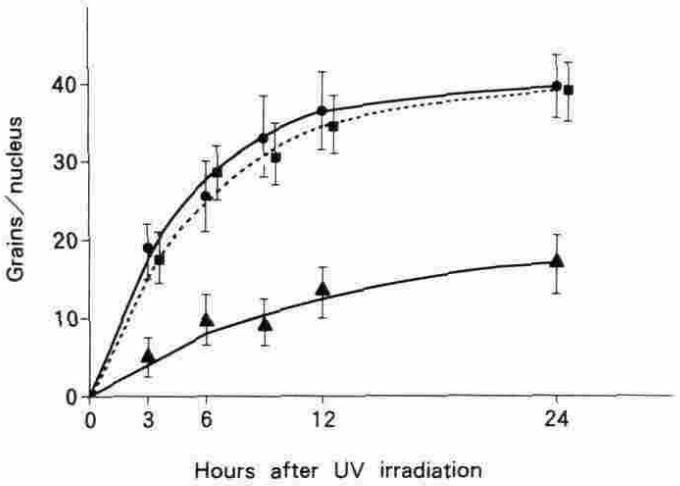

Figure 1. Time kinetics of UV-induced cumulative UDS. Cumulative UDS in terms of mean grains/nucleus was determined autoradiographically at the indicated times during continuous labeling with 370 kBq (10 μCi)/ml of [^{3}H]thymidine after irradiation with 10 J/m^2 UV. *Closed circle:* normal human; *closed square:* XP52KO variant; *closed triangle:* group D XP43KO; *Bar:* SD of the mean.

First we studied the UV dose-dependent production of (6-4) photoproducts in normal, XP35KO (A), XP43KO (D), and XP52KO variant cells by ELISA for the competitive inhibition of 64M-1 monoclonal antibody binding to the standard antigen of high UV dose-irradiated calf thymus DNA by sample ssDNA purified from irradiated cells. The percent inhibition in Fig 2 indicated that the yield of 64M-1 antibody-recognized (6-4) photoproducts in the sample DNA increased linearly with 254 nm UV dose in the range of 0 to 40 J/m^2 254 nm UV. The inhibition was experimentally 0% at 0 J/m^2, although the linear line of the least-squares fit intercepted a 1.4% point of the ordinate at 0 J/m^2 (Fig 2). At 40 J/m^2, competitive inhibition by the irradiated cell DNA was 29.1 $\pm$ 3.1% (Fig 2). Regarding sensitivity, the 64M-1 antibody can sufficiently detect an amount of (6-4) photoproducts produced by at least 10 J/m^2 254 nm UV (Fig 2); that is, approximately 0.002 – 0.006% (6-4) photoproducts per monomeric DNA thymine, as calculated on the assumption that (6-4) photoproducts account for 10% – 30% of cyclobutane dimers (= 0.02%/monomeric thymine at 10 J/m^2 254 nm UV).

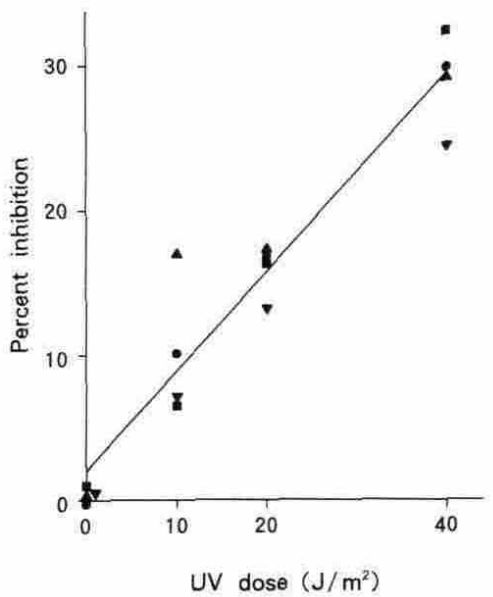

Figure 2. 254-nm UV dose-dependent production of 64M-1 monoclonal antibody-recognizing (6-4) photoproducts as revealed by ELISA. The production was expressed by percent inhibition of 64M-1 binding to the standard antigen (heavily UV-irradiated calf thymus DNA, see *Methods*) by competitive inhibitors of sample cellular DNA from 0 to 40 J/m^2-irradiated cells (see the equation in *Methods*). The line was drawn by the least-squares method. Experimentally, competitive inhibitions were 0% at 0 J/m^2 and 29.1 $\pm$ 3.1% at 40 J/m^2. *Closed circle:* normal human; *closed inverted triangle:* XP35KO (A); *closed triangle:* XP43KO (D); *closed square:* XP52KO variant.

With a fixed 254-nm UV dose of 40 J/m^2 to the DNA in cells, we studied the kinetics of removal of 64M-1 antibody-binding (6-4) photoproduct sites in normal, XP35KO (A), XP43KO (D), and XP52KO variant cells. Figure 3 shows that 64M-1 antibody-binding sites disappeared rapidly from normal cells in such a way that 80%, 90%, and more than 95% of the initial sites were lost in 6, 12, and 24 h, respectively (see also Table I). However, XP35KO (A) cells exhibited no removal of the antibody-binding sites in 24 h, and XP43KO (D) cells had only an approximately 10% loss in 24 h (Fig 3). Our monoclonal antibody results with XP-A and XP-D cells reproduced those that Mitchell et al [13] obtained by a (6-4) photoproduct-specific polyclonal antibody. As described above, XP-A and D cells are defective in the excision repair of both cyclobutane and (6-4) dimers, thus accounting for a high susceptibility to killing, mutagenesis, and skin carcinogenesis. In XP-D cells, however, a 90% inability of excising both cyclobutane and (6-4) dimers does not explain a 20% – 50% high level of early-time UDS in XP-D [1,2], and the 40% cumulative UDS in the present XP43KO (D) (Fig 1).

XP52KO variant cells normally proficient in the ESS or cyclobutane dimer removal (Table I) were only slightly more sensitive ($n = 1.3$, $D_0 = 4.40$ J/m^2) than normal ($n = 1.5$, $D_0 = 5.0$ J/m^2), as shown in Table I, and were sensitive to the synergistic killing effect of post-UV treatment with caffeine (not shown). Mitchell et al [15] have demonstrated immunologically that an XP variant strain is normally proficient in the removal of (6-4) photoproducts. In Fig 3, however, our results showed that XP52KO variant cells had a slightly (20%) retarded removal of the 64M-1 binding sites (see also Table I). Such a small defect in the (6-4) dimer excision in XP variant cells may account for a small hypersensitivity to UV killing (Table I). In conclusion, XP-A and XP-D cells fail to repair both cyclobutane dimers and 64M-1 monoclonal antibody-recognizing (6-4) photoproducts, whereas XP variant cells are weakly defective in only (6-4) photoproduct repair. Further studies on (6-4) photoproduct repair in many XP variant strains will be required to explain the above discordant results of the two XP variant strains.

We first conducted the 254 nm UV experiments as a model. In a view of the relevance of solar UV-B radiation and human skin,

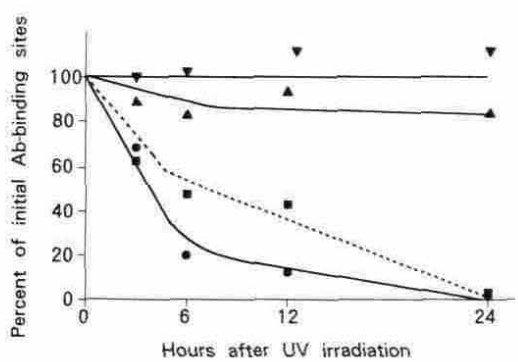

Figure 3. Repair kinetics of the 64M-1 monoclonal antibody-recognizing (6-4) photoproducts. The initial antibody-binding sites of 40 J/m^2-irradiated cellular sample DNA (29.1 $\pm$ 3.1% inhibition in ELISA as shown in Fig 2) was taken as 100%, and a relative decrease in the percent inhibition in ELISA by sample DNA for the presence of repair was plotted as a function of post-UV time. *Closed circle:* normal human; *closed inverted triangle:* XP35KO (A); *closed triangle:* XP43KO (D); *closed square:* XP52KO variant.

however, it is necessary to perform similar experiments using monochromatic 313 nm UV that also produces and degrade (6-4) photoproducts in the DNA.

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